Widespread Muscle Expression of an AAV9 Human Mini-dystrophin Vector After Intravenous Injection in Neonatal Dystrophin-deficient Dogs

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Duchenne (DMD) and golden retriever (GRMD) muscular dystrophy are caused by genetic mutations in the dystrophin gene and afflict striated muscles. We investigated systemic gene delivery in 4-day-old GRMD dogs given a single intravenous injection of an AAV9 vector (1.5×10^{14} vector genomes/kg) carrying a human codon-optimized human mini-dystrophin gene under control of the cytomegalovirus (CMV) promoter. One of the three treated dogs was euthanized 9 days later due to pre-existing conditions. Scattered mini-dystrophin-positive myofibers were seen by immunofluorescent (IF) staining in numerous muscles. At the end of the 16-week study, the other two dogs showed generalized muscle expression of mini-dystrophin in ~15% to nearly 100% of myofibers. Western blot and vector DNA quantitative PCR results agreed with the IF data. Delayed growth and pelvic limb muscle atrophy and contractures were seen several weeks after vector delivery. T-2 weighted magnetic resonance imaging (MRI) at 8 weeks showed increased signal intensity compatible with inflammation in several pelvic limb muscles. This marked early inflammatory response raised concerns regarding methodology. Use of the ubiquitous CMV promoter, extra-high vector dose, and marked expression of a human protein in canine muscles may have contributed to the pathologic changes seen in the pelvic limbs.

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder affecting ~1 of 3,500 newborn human males in whom absence of the protein dystrophin causes progressive degeneration of skeletal and cardiac muscles.[1] No treatment halts or reverses progression of DMD. Although cellular and gene therapies are promising, key questions must first be addressed in relevant animal models. Spontaneous forms of X-linked muscular dystrophy due to dystrophin deficiency have been identified in mice,[2,3] multiple dog breeds,[4–7] and cats.[8,9] Unlike the dystrophin-deficient mdx mouse, which shows relatively mild clinical signs, affected dogs develop progressive, fatal disease strikingly similar to the human condition. Accordingly, studies in the canine dystrophin-deficient models, such as golden retriever muscular dystrophy (GRMD), may be more likely than those in mdx mice to predict pathogenesis and treatment outcome in DMD.

Adeno-associated virus (AAV)–mediated mini- and micro-dystrophin gene therapy has shown promise in mdx mice, with widespread expression demonstrated after systemic delivery in neonatal[10] and older mice with chronic disease.[11] However, use of AAV-mediated gene therapy in murine models of other diseases such as hemophilia has not consistently predicted the degree of immunologic response.[12] In keeping with this species dichotomy and in contrast to findings in mdx mice, studies of localized (intramuscular) AAV-mediated dystrophin mini (or micro) gene therapy in GRMD dogs have documented a marked immune response to either components of the transgene (to include dystrophin)[13] and/or viral capsid proteins.[14,15] Because of the relative immaturity of the neonatal immune system, animals treated soon after birth may mount a less robust response. Indeed, long-term, widespread muscular transduction with a human placental alkaline phosphatase reporter gene has been reported after systemic intravenous delivery of an AAV9 construct in normal, neonatal dogs.[16]

Here, we report findings from three GRMD dogs that were administered an AAV9-CMV-human mini-dystrophin construct intravenously at 4 days of age. Although widespread muscle expression of mini-dystrophin was seen 16 weeks after treatment, the affected dogs also had pelvic limb muscle atrophy and contractures, apparently associated with an early innate immune response.

RESULTS

Clinical and pathologic findings in individual dogs

All three GRMD dogs were homozygous females. Each had elevation of serum creatine kinase on the day of birth: RaF7 (>300,000 U/l), Emerald (241,200 U/l), and Amethyst

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(294,000 U/l). One of the dogs (RaF7) was lethargic and stunted (birth mass of 194 g compared to 275–290 g for its four GRMD littermates) prior to vector injection and was euthanized 9 days later due to persisting lethargy and anorexia. On histologic evaluation of the liver, there was marked steatosis confined to the left medial lobe, most likely due to a congenital condition unrelated to vector delivery. The rest of the liver was grossly and histologically normal (**Supplementary Figure S1**).

The other two treated homozygous female GRMD dogs (Emerald and Amethyst) had delayed growth compared to two untreated GRMD male littermates (Jasper and Peridot) and two untreated homozygous affected females born in a different litter 3 days later (Hope and Vasalia) (**Supplementary Figure S2**). Although all six dogs were of near equal size at birth, by 16 weeks, the two treated females weighed considerably less than the untreated dogs [note that GRMD males typically weigh ~15% more than homozygous GRMD females at 6 months (J.N. Kornegay and D.J. Bogan, unpublished results)]. Emerald and Amethyst developed pelvic limb muscle atrophy and contractures that were most pronounced in the right pelvic limb of Emerald. The stifle (knee) joint was locked in extension, consistent with quadriceps contracture seen in dogs with inflammatory neuromyopathies.[17]

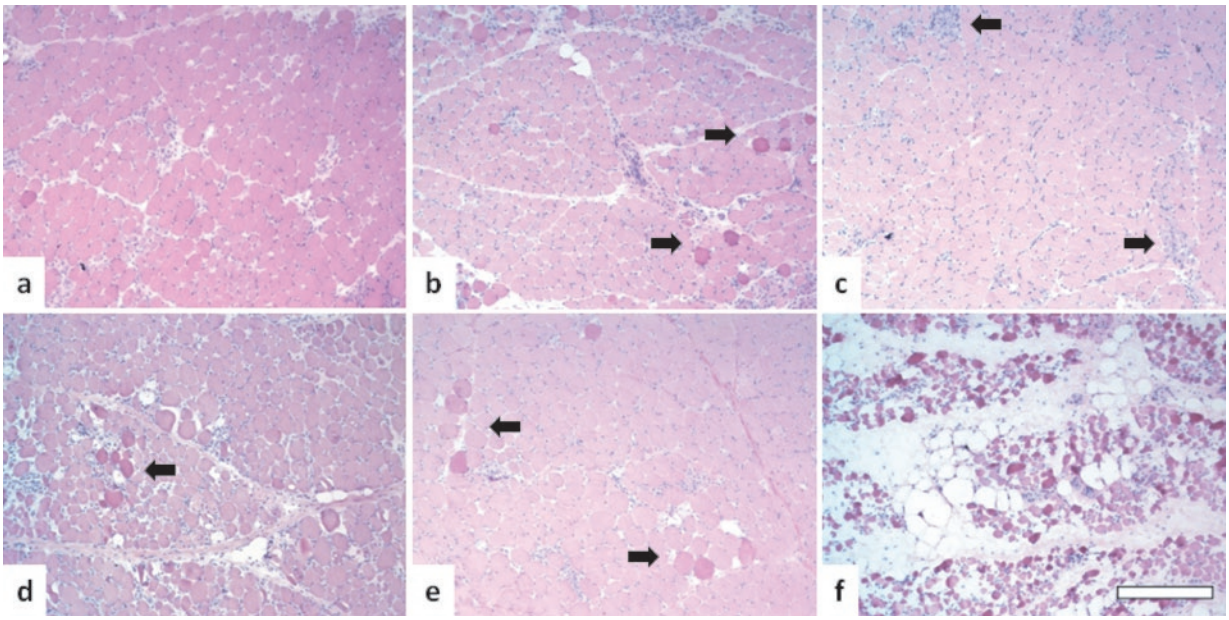

Figure 1 Histopathological changes in muscles 16 weeks after AAV9-CMV-mini-dystrophin vector intravenous injection of golden retriever muscular dystrophy (GRMD) dogs Emerald (E) and Amethyst (A) at 4 days of age. (**a**) Semitendinosus (E), (**b**) psoas major (E), (**c**) peroneus longus (A), (**d**) diaphragm (E), (**e**) cranial tibial (A), and (**f**) vastus medialis (E). All muscles have changes typical of GRMD, including small group myofiber necrosis (arrows in **b**, **d**, and **e**) and regeneration (arrows in **c**). Muscle is otherwise relatively normal histologically in all but the vastus medialis (**f**) in which marked deposition of fat is evident. Hematoxylin and eosin; bar = 300 µm.

Table 1 Serum chemistry values

Test/ week	Emerald				Amethyst				Visalia				Hope				Bryce			
	d0	4	8	12	0	4	10	12	0	4	8	12	0	4	8	12	0	4	8	12
CK (U/l)	241,200	4,500	12,450	8,480	294,000	313	5,680	23,120	90,400	14,080	11,100	11,600	137,000	689	42,900	2,630	3,000	316	313	453
BUN (mg/dl)	18	8	12	11	23	6	13	16	23	8	3	7	20	9	6	7	23	7	7	9
GGT (U/l)	7	16	16	16	NA	9	6	16	42	16	16	9	NA	9	8	8	68	20	7	9
ALT (U/l)	112	177	242	364	225	106	298	415	227	171	310	311	193	151	313	278	29	8	54	38
AST (U/l)	890	79	142	299	2,548	78	351	441	1,100	323	408	355	650	151	402	633	142	11	26	19
Alk phos (U/l)	532	122	58	62	624	129	97	91	656	96	73	48	871	144	89	64	703	148	141	162
Total Bili (mg/dl)	1.2	0.4	0.5	0.6	1.0	0.5	0.5	0.5	1.7	0.3	0.6	0.6	NA	0.3	0.8	0.8	0.7	0.5	0.7	0.8
Total protein (g/dl)	3.8	4.0	5.4	5.5	4.2	4.1	5.6	5.9	4.5	4.1	6.6	4.8	4.4	3.8	6.1	NA	3.7	4.1	4.7	6.0

Emerald, Amethyst, Visalia, and Hope are all homozygous female GRMD dogs; Bryce is a normal male dog. An 8-week sample was not available for Amethyst.
Alk phos, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Bili, bilirubin; BUN, blood urea nitrogen; CK, creatine kinase; GGT, gamma-glutamyl transferase; NA, not applicable.

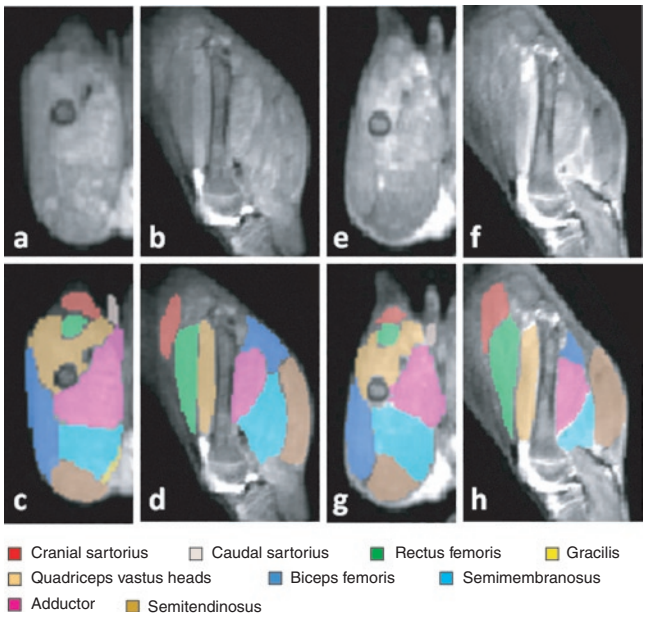

Figure 2 T2-weighted magnetic resonance images of pelvic limb muscles 8 weeks after AAV9-CMV-mini-dystrophin vector intravenous injection of golden retriever muscular dystrophy dogs Emerald and Amethyst at 4 days of age. Transverse (left) and sagittal (right) images of (**a**–**d**) Amethyst and (**e**–**h**) Emerald are seen. The images in **c**,**d**,**g**, and **h** have been segmented and color coded to outline individual muscles. Signal intense lesions are particularly pronounced in the vastus heads of the quadriceps and adductor muscles. These changes persisted with fat saturation suggesting that they most likely represent fluid due to inflammation or edema.

Both dogs were active and alert despite their impaired pelvic limb function. Serum chemistries of all four dogs at 1, 2, 4, 6, 8, and 12 weeks were largely unremarkable, except for elevation of creatine kinase, alanine aminotransferase, and aspartate aminotransferase (**Table 1**), which are associated with muscle necrosis in GRMD.[18] On electromyography at 16 weeks, there were pronounced complex repetitive discharges in pelvic limb muscles, consistent with prior reports,[4,19] but no evidence of denervation in the pelvic limb muscles. Nerve conduction velocity was normal (data not shown).

Emerald and Amethyst were euthanized at 16 weeks. At necropsy, the quadriceps femoris muscle of Emerald was atrophied and the stifle (knee) could not be flexed. Characteristic small group myofiber necrosis and regeneration was present in all muscles. However, histopathological changes were relatively mild in all but the quadriceps muscle in which there was marked fatty deposition, especially in the vastus medialis head (**Figure 1**).

MRI

T2-weighted magnetic resonance imaging (MRI) 8 weeks after perfusion showed increased signal intensity in several proximal pelvic limb muscles of Emerald and Amethyst (**Figure 2**). The quadriceps femoris and adductor muscles were particularly involved in both dogs. Individual heads of the quadriceps were poorly defined, but signal intensity was most pronounced in the vastus intermedius and medialis. These increases in signal intensity persisted with fat saturation in keeping with a fluid signal most likely associated with edema due to inflammation. MRI findings were largely consistent with histopathological changes.

Analyses of mini-dystrophin gene expression in various muscle groups

Immunofluorescent staining for mini-dystrophin was done in the muscles of all three treated dogs. In RaF7, who was euthanized 9 days after vector delivery, scattered mini-dystrophin transgene-positive myofibers were observed in numerous muscle groups (**Supplementary Figure S3**), indicating that widespread mini-dystrophin gene expression occurred as early as 9 days after intravenous AAV9 injection. In Emerald and Amethyst, who were euthanized 16 weeks after vector delivery, widespread and high-level expression of mini-dystrophin was observed in multiple muscles, ranging from ~15 to nearly 100% of the myofibers being positive for mini-dystrophin staining (**Figure 3a,b**). Areas in which there was near uniform mini-dystrophin expression did not have dystrophic lesions and no inflammatory cell infiltrate (**Figure 4**). Western blot analysis (**Figure 5**) and quantitative PCR on vector DNA (**Figure 6**) were generally consistent with the immunofluorescent (IF) results. Interestingly, pelvic limb muscles with a lower percentage of mini-dystrophin-positive myofibers by IF staining (e.g., rectus femoris) had an even higher quantity of the protein on western blot than the thoracic limb muscles (e.g., long head triceps), which had nearly 100% positive myofibers (comparing **Figures 5** and **6**), suggesting that individual mini-dystrophin-positive myofibers in the pelvic limb muscles had higher transgene expression.

DISCUSSION

These results support the feasibility of systemic intravenous delivery of truncated forms of dystrophin to skeletal muscles of neonatal GRMD dogs by AAV vectors. There was widespread transduction of skeletal muscles in the two dogs, Emerald and Amethyst, with nearly 100% of myofibers of some muscles being positive for mini-dystrophin gene expression 16 weeks after perfusion (duration of study). Although the long-term expression of mini-dystrophin is encouraging, the apparent myositis with contractures in these two dogs raises questions about the relative roles that AAV capsid antigens or the transgene itself may have played to induce an immune response. Others have documented a marked inflammatory response to either the transgene[13] and/or viral capsid proteins[14,15] in GRMD dogs injected intramuscularly with AAV-micro-dystrophin constructs. The immune responses were T-cell mediated and eventually eliminated micro-dystrophin expression in the vector-injected muscles. An analogous humoral immune response to the transgene has been seen in hemophiliac dogs following intramuscular injection of AAV2-Factor IX constructs.[20] With both the hemophiliac and GRMD dogs, the immune response was blunted with transient immunosuppression.[20,21]

Immune function has traditionally been divided between innate and adaptive components. The innate immune system is generally thought to provide an immediate, nonspecific response to foreign antigens that confers no lasting immunity. In contrast, adaptive immunity is delayed, requires activation of specific clones of lymphocytes, and leads to persisting immunity.[22–24] The lack of a sustained immune reaction to the AAV9-mini-dystrophin

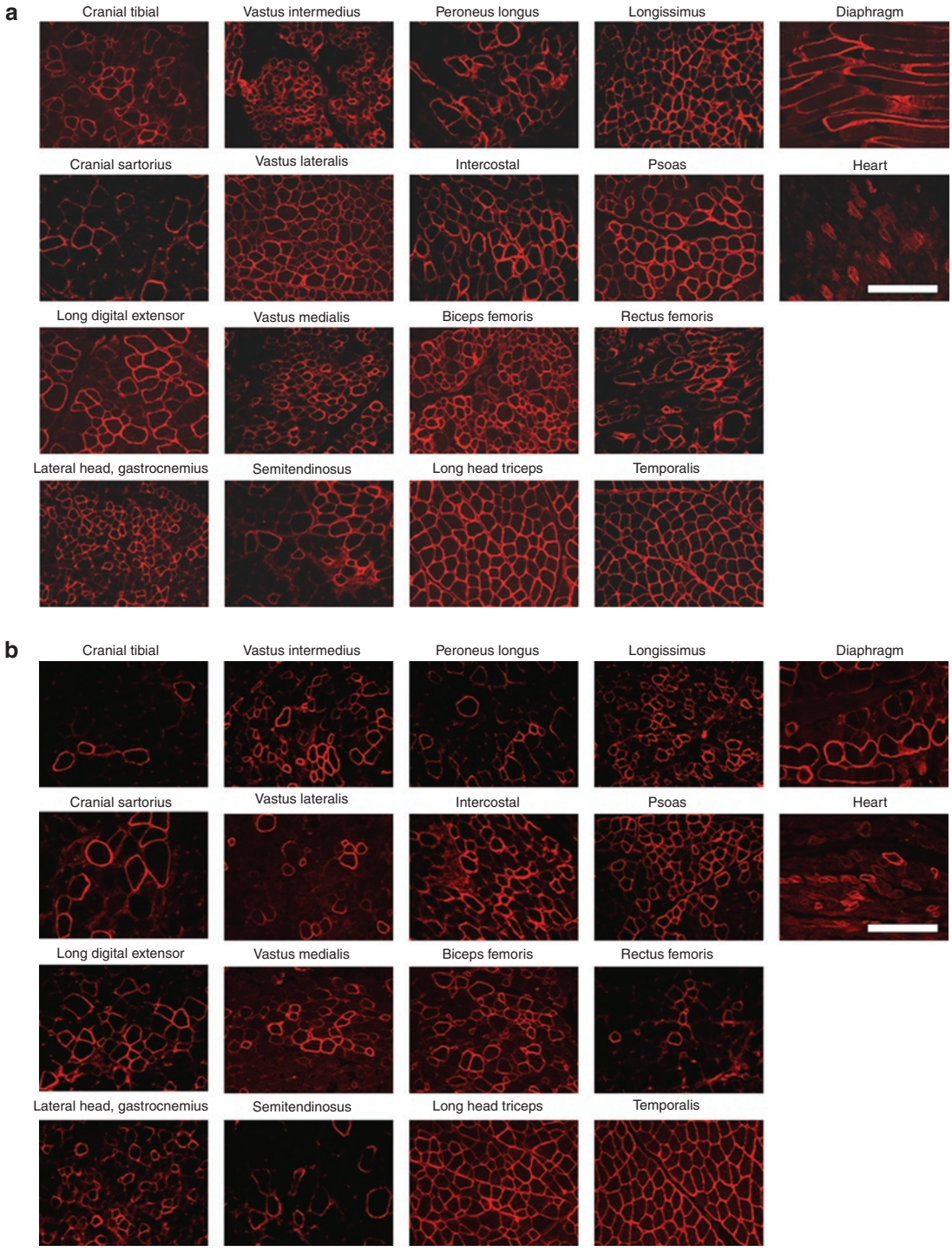

Figure 3 Body-wide extensive human mini-dystrophin expression 16 weeks after intravenous injection of AAV9-CMV-mini-dystrophin vector in golden retriever muscular dystrophy dogs (a) Emerald and (b) Amethyst at 4 days of age. Cryo-thin sections of the muscle and heart were stained by immunofluorescence with antibody against human mini-dystrophin (red color) and counterstained with DAPI to show cell nuclei (blue color). Variable and extensive gene expression in multiple skeletal muscles is readily visible. Photomicrographs were taken from the areas of best mini-dystrophin expression in each muscle. **a**,**b**, Bar = 250 µm.

construct in these dogs argues against a specific adaptive immune reaction. Factors contributing to long-term mini-dystrophin expression in these dogs could include the relative immaturity of the neonatal immune system, lower antigen loads in muscle with systemic versus localized vector administration, and the potential for a lower immune profile with AAV9. Unlike rodents, the immune system of humans and dogs is essentially mature at birth.[25] However, neonatal dogs have lower immunoglobulin levels and fewer T versus B lymphocytes. Moreover, their peripheral blood lymphocytes respond less vigorously to antigens *in vitro*.

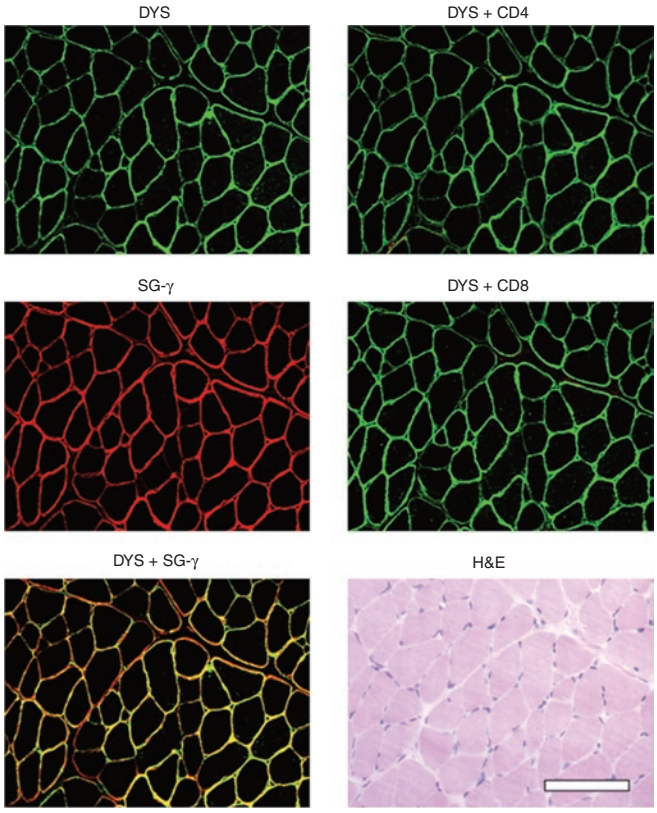

Figure 4 Lack of immune infiltration and dystrophic lesions in muscles with uniform human mini-dystrophin expression 16 weeks after intravenous injection of AAV9-CMV-mini-dystrophin vector in golden retriever muscular dystrophy dog Emerald at 4 days of age. The long head of the triceps muscle of Emerald was cryo-thin-sectioned. Consecutive sections were subjected to H&E and fluorescent staining with antibodies against mini-dystrophin (DYS, green color), sarcoglycan-γ (SG-γ, red color), CD4 (red color), and CD8 (red color) T cells. Note the lack of T-cell infiltration and dystrophic lesions in the muscle. Bar = 150 µm. H&E, hematoxylin and eosin.

Taken together, these differences could dampen the immune response of neonatal dogs to antigens such as AAV or mini-dystrophin. Prior studies of the immunogenicity of AAV-mini-dystrophin constructs in dogs have focused on intramuscular delivery.[13–15,21] Studies have also demonstrated long-term expression of Factor IX in dogs following intramuscular injection of AAV constructs.[20,26] Although such localized approaches reduce the risk of systemic side effects, an immune response may be more likely in muscle, simply because of the concentrated antigen load. Other studies of AAV in GRMD and hemophiliac dogs have mostly used either AAV2 or 6, although AAV9 was employed in dogs of this report and an earlier study in which the human alkaline phosphatase gene was expressed in neonatal dogs.[16] In general, AAV capsid antigens are remarkably conserved across serotypes,[27] with the AAV8 capsid amino-acid sequence being 83% conserved compared to AAV2. Other distinguishing features such as the kinetics of vector uncoating also influence the immune response. AAV2 slowly uncoats, thus potentially allowing more time for capsid peptides to be presented to immune cells.[28] On the other hand, based on results from hemophiliac dogs, the fact that AAV2 transduces muscle less efficiently than certain AAV serotypes reduces expression of Factor IX and the associated immune response.[12] Although studies in mice and nonhuman primates have suggested that AAV serotypes that do not bind heparan sulfate proteoglycans are less able to activate T cells after intramuscular injection,[29] use of the nonheparin-binding AAV1 serotype did not eliminate immunogenicity in GRMD dogs.[15]

Just as multiple factors may have allowed long-term expression of the transgene in these dogs, several elements potentially contributed to the inflammatory reaction. Both the AAV9 vector and the transgene should be considered. It is unlikely that the immune response was triggered by protein impurities in the AAV9 vector preparations. Protein gel examination showed that the final vector preps used in the *in vivo* study were >95% pure (**Supplementary Figure S4**). Nonetheless, we cannot completely rule out the possibility that vector impurities contributed to the response. While pre-existing immunity to AAV antigenic epitopes could have led to the immune response, our prior studies have found no such antibodies in naive dogs of various ages in the colony (data not shown). The 10–60% homology between the canine parvovirus capsid protein and that of AAV6 could predispose dogs to an immune reaction. But, others have shown that vaccination with canine parvovirus does not induce a humoral immune response to AAV.[15] In their hands, there was limited or no homology between canine parvovirus and the epitopes of AAV6 that were identified to be immunogenic. Another study used AAV9 vector to express the human placental alkaline phosphatase reporter gene under transcriptional control of the ubiquitous Rous sarcoma virus promoter in normal neonatal dogs without side effects.[16] Separately, we have also used an AAV9 vector to express either canine mini-dystrophin (two dogs; 4 months old) or codon-optimized human mini-dystrophin (one dog; 2 months old), both under control of the human cytomegalovirus (CMV) promoter.[30] Each construct was injected intravenously into a pelvic limb isolated from the general circulation using a tourniquet in the inguinal area (hydrodynamic limb perfusion). Widespread mini-dystrophin expression was observed in all three dogs at the time of the last biopsy at 1 year, 1.5 years, and 2 years after perfusion (J. Li, J.R. Bogan, D.J. Bogan, J.N. Kornegay, and X. Xiao, unpublished results), again arguing against cellular immune responses toward the vector-transduced myofibers. However, transient inflammation was observed in the limb that was perfused with the human codon-optimized human mini-dystrophin, suggesting this as the source of the immune reaction. We had previously largely shifted to canine mini-dystrophin but chose to use the human version in these dogs for the sake of the codon optimization, which generated approximately tenfold higher gene expression than the nonoptimized one when examined in mdx mice. Besides the potential antigenicity of the human mini-dystrophin, the remarkably high vector dose (1.5×10^{14} viral genomes/kg) and greatly enhanced expression due to codon optimization may have led to extremely high-level expression in some muscles such as the quadriceps and also in nonmuscle tissues, thus triggering an innate immune response. Use of the same vector and dose caused similar side effects in neonatal dystrophin–utrophin double knockout mice with an immature immune system. A tenfold lower dose rendered profound therapeutic benefits in these mice without apparent side effects (P. Hu, B. Wang, J. Li, and X. Xiao, unpublished results). Although an adaptive cellular immune response could not be ruled

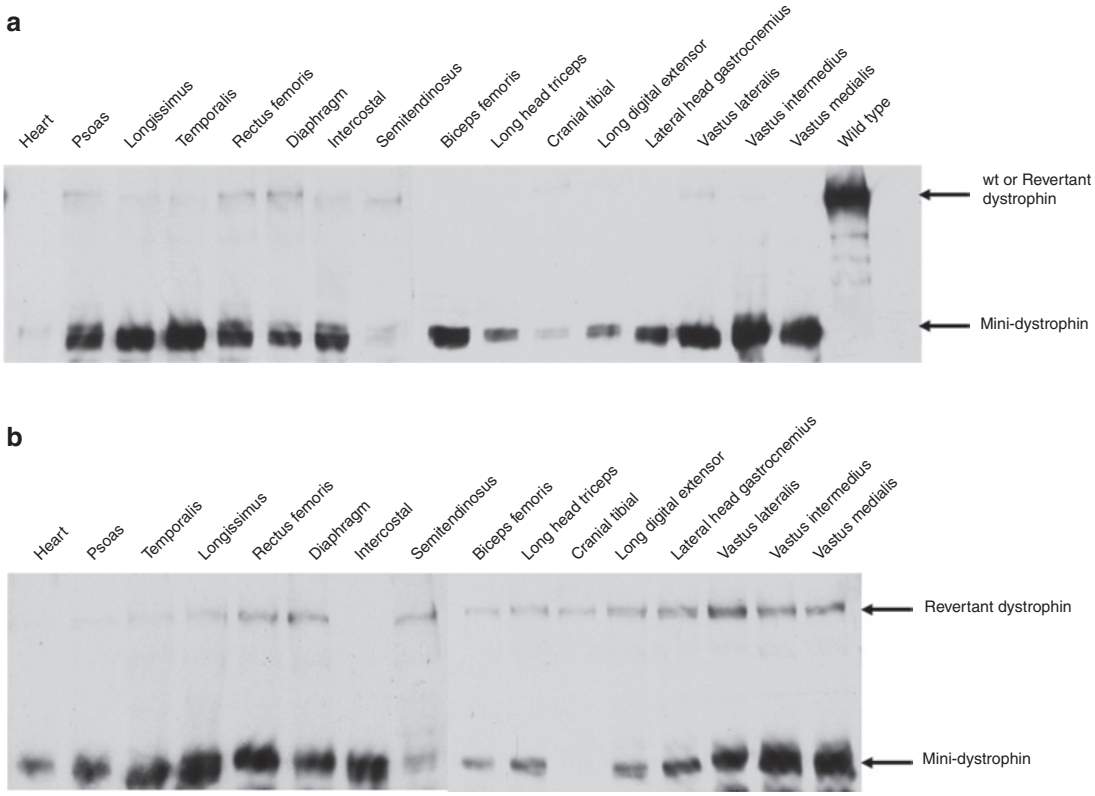

Figure 5 Analyses of mini-dystrophin expression by western blot in muscle and heart samples at necropsy 16 weeks after intravenous injection of AAV9-CMV-mini-dystrophin vector in golden retriever muscular dystrophy dogs Emerald and Amethyst at 4 days of age. Human mini-dystrophin and low levels of endogenous revertant dystrophin are seen in nearly all skeletal muscles. Low levels of human mini-dystrophin are seen in the left ventricle of the heart, whereas revertant dystrophin is undetectable. (**a**) Emerald and (**b**) Amethyst.

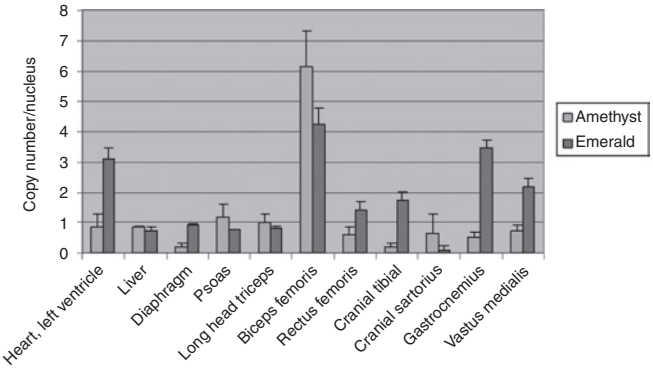

Figure 6 Analysis of AAV vector DNA by quantitative PCR in muscle and heart samples at necropsy 16 weeks after intravenous injection of AAV9-CMV-mini-dystrophin vector in golden retriever muscular dystrophy dogs Emerald and Amethyst at 4 days of age. Numbers are shown on a per nucleus (diploid genome) basis. The data were obtained by dividing total vector copy numbers by 2 × the total copy numbers of an endogenous single-copy gene glucagon in the PCR reactions of each muscle sample.

out in the GRMD dogs reported here, the long-term body-wide human mini-dystrophin expression and lack of T-cell infiltration are more consistent with innate immunity. The precise underlining mechanisms remain elusive and require further study.

The clinical syndrome of contractures and associated MRI findings in these dogs warrants special comment. Inflammatory muscle disease characteristically causes signal intense lesions on T2-weighted MR images.[31,32] These changes do not suppress with fat saturation and are thought to reflect a fluid signal subsequent to edema. Somewhat analogous but less severe lesions occur in GRMD muscle independent of treatment, presumably because of inflammation and edema associated with muscle necrosis,[33] and have also been reported in GRMD dogs treated with AAV vectors.[15] The preferential involvement of the vastus heads of the quadriceps femoris and adductor muscles is in keeping with selective involvement of certain muscles in inflammatory myopathies in both humans and dogs. In particular, the vastus heads of the quadriceps are dramatically affected despite sparing of the rectus femoris in humans with sporadic inclusion body myositis.[34] Similarly, some dogs with *Neospora caninum* infection develop hyperextension of the stifle (knee) (genu recurvatum) secondary to quadriceps myositis and contractures akin to those seen in Emerald,[17] suggesting that those muscles are more sensitive to inflammation. Reasons for this predominant involvement of particular muscles have not been defined in either disease.

Studies of gene therapy for DMD have been facilitated by the availability of both small (mdx) and large (GRMD) animal models. Results from mdx mice have largely supported application to human patients, although those from GRMD have identified risks of immune rejection. Our findings support the potential use of systemic gene therapy in DMD but also point to potential risks. A somewhat similar paradigm exists with hemophilia where AAV

constructs have generally induced a more pronounced immune response in dogs.[12] With this said, canine hemophilia studies did not predict a T cell–mediated response to AAV capsid antigens that destroyed transduced hepatocytes in a human trial.[35] Further experiments in the GRMD model are needed to define the nature of the immune response to AAV-mini-dystrophin constructs.

MATERIALS AND METHODS

Animals. This study involved three homozygous female GRMD dogs produced in a colony at the University of North Carolina at Chapel Hill (UNC-CH). Dogs were used and cared for according to principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The phenotype was initially determined based on the elevation of serum creatine kinase and confirmed by PCR.

At 4 days of age, the three dogs weighed from 243 to 450 g. Butterfly catheters were placed in the jugular veins. Codon-optimized, AAV9-CMV-human mini-dystrophin construct (1.5×10^{14} vg/kg) was suspended in 15 ml/kg of saline (total volume of 3.65–6.75 ml) and administered at a rate of ~1 ml/minute. The dogs were observed and weighed daily for the first 6 weeks and weekly thereafter.

Mini-dystrophin gene in AAV9 vectors. The human mini-dystrophin gene (DysΔ3990), as reported previously,[36] was modified by human codon usage optimization and fully synthesized (GeneArt, Toronto, Ontario, Canada). The optimized mini-dystrophin, named opti-DysΔ3978, encodes the same protein as its precursor but with optimized DNA sequences for better RNA processing and protein translation. The opti-DysΔ3978 mini-dystrophin gene was subcloned into an AAV expression vector cassette under transcriptional control of a CMV promoter for strong expression.[36] The opti-DysΔ3978 mini-dystrophin gene expression vector was then packaged into AAV9 capsids[37] and purified by double CsCl density ultracentrifugation using previously published adenovirus-free, triple plasmid transfection protocols.[38] The vector titers were determined by DNA dot blot at ~1×10^{13} viral genome particles per ml in 1× phosphate-buffered saline solution plus 3% sorbitol.

IF staining and western blot. IF staining of mini-dystrophin in the cardiac and skeletal muscles of the GRMD dogs after AAV9 vector gene transfer was performed similarly using a protocol described previously.[36] Briefly, the snap-frozen tissues were cryo-thin-sectioned at 8 µm thickness and blocked with 5% horse serum in phosphate-buffered saline without prior fixation. Polyclonal primary antibodies were used for immunostaining of dystrophin (anti-Rod1 and Rod2 regions of human dystrophin 1:500). Monoclonal primary antibodies were used for the immunostaining of γ-sarcoglycans at 1:100 dilution (NCL-g-SARC; Novocastra, Burlingame, CA). IF staining of canine $CD4^+$ and $CD8^+$ T cells was performed by monoclonal antibodies, rat anti-canine CD4 and CD8 IgGs at 1:100 dilution (Serotec, Oxford, UK). Two different fluorophore-labeled secondary antibodies were used in this study: red fluorescence (Cy3-conjugated AffiniPure goat anti-rabbit IgG, and Cy3-conjugated goat anti-mouse IgG and goat anti-rat IgG; Jackson ImmunoResearch, West Grove, PA) and green fluorescence (Alexa Fluor 488 chicken anti-rabbit IgG or goat anti-mouse IgG; Molecular Probes, Eugene, OR). All were diluted with 5% horse serum in 1× phosphate-buffered saline at 1:500 of ratio for use.

Western analysis for mini-dystrophin expression in dog muscle. Western analysis was carried out according to previously published methods.[39,40] Briefly, 20 cryosections of each muscle sample were lysed in 200 µl of protein lysis buffer with proteinase inhibitor cocktail (Sigma-Aldrich, St Louis, MO). After brief sonication and vortexing, the lysate was spun at 12,000 rpm in 4 °C for 10 minutes and supernatant collected. The protein concentration was determined by the Bradford method (Bio-Rad protein assay; Bio-Rad Laboratories, Hercules, CA). A total of 20 µg protein per sample was boiled with sample loading buffer. The samples were separated by 5% SDS-PAGE and electrotransferred to PVDF membranes. After blocking in 10% nonfat dry milk in TBS buffer (50 mmol/l Tris-Cl, pH 7.5, 200 mmol/l NaCl) for 1 hour, the membranes were incubated with primary antibodies in TBS containing 0.5% Tween-20 (TTBS) at room temperature for 1 hour. A rabbit polyclonal antibody recognizing human dystrophin rod 22 and rod 23 regions was used in this experiment with a 1:5,000 dilution. Following primary antibody incubation and multiple rinses, the membranes were incubated with the secondary antibody, a goat anti-rabbit conjugated with horseradish peroxidase (Sigma-Aldrich) with 1:5,000 dilution in 2% dry milk and TBS buffer. After 1-hour antibody incubation and three washes with TTBS buffer and once with TBS, the full-length dystrophin from normal dog muscle and AAV vector-derived human mini-dystrophin protein bands were visualized with chemiluminescence reagent (DuPont NEN, Boston, MA) by exposure to X-ray film.

MRI. At 8 weeks after perfusion, dogs were premedicated with acepromazine maleate (0.2 mg/kg), butorphanol (0.4 mg/kg), and atropine sulfate (0.04 mg/kg), masked, and then intubated and maintained with isoflurane. Anesthetized dogs were positioned in ventral recumbency (prone position) in a Siemens 3T Allegra Head-Only MRI scanner (Siemens, Erlangen, Germany) and Siemens standard CP Head Coil (Siemens). The pelvic limbs were extended caudally and positioned in the head coil centered at midfemur. An initial FISP pulse sequence was performed to localize the sagittal and transverse planes. Transverse images were then collected using a 3D TSE VFL sequence (TR 3,000 ms, TE 409 ms, FOV 230 × 210 mm). A total of 160 slices (0.9 mm; voxel size 0.9 mm^3 for Emerald and 0.7 mm^3 for Amethyst) were collected extending from the hip to stifle joints. Images were collected with and without fat saturation. The scan time for each run was 11:38. The 3DT2 and 3DT2FS transverse images were reconstructed in the transverse orientation using the standard Siemens 3D MPR application. Raw and reconstructed 3DT2 and 3DT2FS images were transferred to the image PACS system for download and image analysis.

SUPPLEMENTARY MATERIAL

Figure S1. Photomicrographs from (**a**) grossly normal and (**b**) pale lobes of the liver of GRMD RaF7 9 days after AAV9-CMV-mini-dystrophin vector intravenous injection at 4 days of age.

Figure S2. Body mass (g; vertical axis) versus age (weeks; horizontal axis) at 2-week intervals from birth to 16 weeks of two GRMD dogs (Emerald and Amethyst) after intravenous injection of AAV9-CMV-mini-dystrophin vector at 4 days of age and two untreated male littermate GRMD dogs (Peridot and Jasper) and two untreated homozygous GRMD females (Hope and Vasalia) born 3 days later.

Figure S3. Human mini-dystrophin expression in multiple muscles 9 days after AAV9-CMV-mini-dystrophin vector intravenous injection of GRMD dog RaF7 at 4 days of age.

Figure S4. AAV9 viral vector purity examined by polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining.

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